STUDIES ON AMINO-ACIDS AND RELATED COMPOUNDS. PART II. ELECTROLYTIC OXIDATION OF PYRROLIDONE-CARBOXYLIC ACID.

By Yoshitaro TAKAYAMA.

Received March 27th, 1933. Published May 28th, 1933.

In the previous communication,[1] it was stated that succinamic acid was obtained from the electrolysate of glutamic acid, and its formation was ascribed to the anodic oxidation of pyrrolidone-carboxylic acid, which could be formed, apart from electrolysis, by the dehydration of glutamic acid.

The formation of pyrrolidone-carboxylic acid from glutamic acid is well known. Menozzi and Appiani[2] first stated that glutamic acid is very easily converted by heating into its lactam, pyrrolidone-carboxylic acid. E. Abderhalden[3] reported that *l*- and *dl*-pyrrolidone-carboxylic acids were obtained from glutamic acid when it was heated to 150–160°C. and 180–190°C. respectively. F. W. Foreman[4] observed that glutamic acid was converted into its lactam when its aqueous solution was boiled for a certain length of time, and reversely, pyrrolidone-carboxylic acid was reconverted into glutamic acid by boiling with strong hydrochloric acid. C. Okinaka[5] found that above reversible reaction occurred even in the absence of acid or alkali, and studied the equilibrium points of above reversible reactions at 120°, 130° and 140°C. He also mentioned that the rate of transformation into the lactam was increased as the temperature was raised or by the presence of dilute alkali, and it was retarded in the presence of an excess of strong acids or alkalies.

The author intended to ascertain that such a reversible reaction would occur actually even in the solution in normal sulphuric acid and at a lower temperature, and has undertaken the following experiments.

d-Glutamic acid and *l*-pyrrolidone-carboxylic acid were dissolved in N.-sulphuric acid, and kept at 35° and 50°C., and amounts of amino-N in their solution were determined from time to time. The changes of amino-N

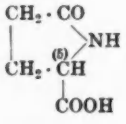
(1) Y. Takayama, Studies on amino-acids and related compounds. Part I.
(2) Menozzi und Appiani, Abderhalden: „Biochem. Handlexikon" **4**, 615.
(3) E. Abderhalden und K. Kautzsch, *Z. physiol. Chem.*, **64** (1910) 447.
(4) F. W. Foreman, *Biochem. J.*, **8** (1914), 481.
(5) C. Okinaka, "The Sexagint" (a collection of papers dedicated to Prof. Y. Osaka by his pupils in celebration of his 60th birthday) Kyoto, (1927), p. 27.

content thus obtained showed the degree of their conversions. The results obtained are tabulated in Fig. 1, Tables I and II.

From the above results it is clear that the reversible reaction proceeds even at such a low temperature as 35°C., and we can conclude that a solution of glutamic acid, kept under similar conditions as in the electrolytic oxidation, should contain a certain amount of pyrrolidone-carboxylic acid (i).

Next, in order to compare the rate of the electrolytic oxidation of glutamic acid with that of pyrrolidone-carboxylic acid, each of these acids was dissolved in N.-sulphuric acid and oxidised with a lead peroxide anode under the same conditions. The amounts of ammonia-N formed were determined at a certain interval of time. The results are given in Fig. 2, the curves representing ammonia-N/faradays per mol.

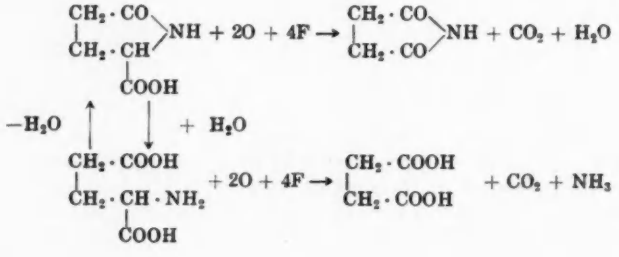
From these curves we see that the rate of oxidation of pyrrolidone-carboxylic acid is far greater than that of glutamic acid and the difference of the rates may be ascribed to the presence of the easily oxidisable carbon (5) in the former acid (ii).

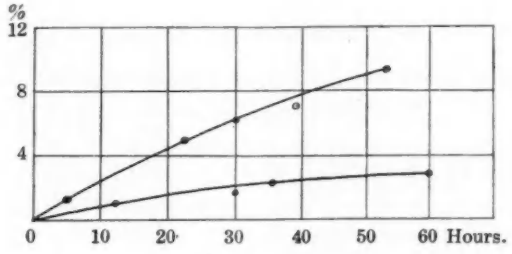


Pyrrolidone-carboxylic acid was dissolved in N.-sulphuric acid and oxidised at a lead peroxide anode. The oxidised solution contained a little of ammonia-N (MgO) and much of ammonia-N (NaOH), hence the presence of imino or amino compound is easily presumable (iii).

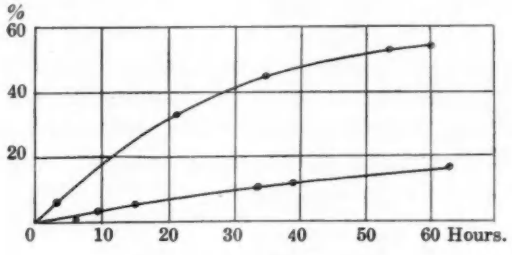
In fact, succinimide was isolated abundantly together with a small amount of succinic acid (iv).

From these facts, (i), (ii), (iii) and (iv), it is quite probable that the oxidation of pyrrolidone-carboxylic acid proceeds in the following scheme and that succinamic acid formed from glutamic acid is produced by the hydrolysis of sccinimide.



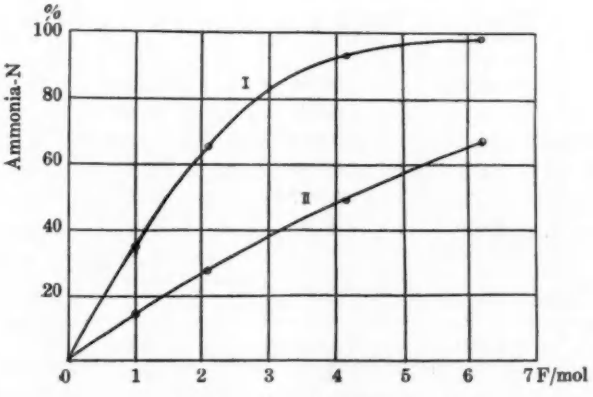


Glutamic Acid Changed.



Pyrrolidone-Carboxylic Acid Changed.

Fig. 1.



I = Pyrrolidone-Carboxylic acid.
II = Glutamic acid.

Fig. 2.

Experimental Part.

Conversion of Glutamic Acid into Pyrrolidone-Carboxylic Acid and vice versa.

d-Glutamic acid (N = 9.25%; calc. N = 9.50%)
$[\alpha]_D^{20} = +30.5°$ (as hydrochloride).
l-Pyrrolidone-carboxylic acid (N = 10.67%; calc. N = 10.85%)
$[\alpha]_D^{20} = -11.55°$ (aqueous solution).

The solutions used: 5 gr. of glutamic acid in 50 c.c. of N.-sulphuric acid, and 5 gr. of pyrrolidone-carboxylic acid in 50 c.c. of N.-sulphuric acid.

The procedure: The solution to be examined was introduced into a stoppered flask and kept at 35° or 50°C. in a thermostat. After a certain interval of time, 2–5 c.c. of the solution were taken, and total-N as well as amino-N (van Slyke) in it were determined. The results of the experiment are shown in Tables I and II.

Table I.

Time.	Total-N. in 100 c.c.	NH_2-N. in 100 c.c.	Glutamic acid.	Glutamic acid changed.
Glutamic acid at 50°C.				
0 hrs.	0.8762 gr.	0.8694 gr.	99.2%	0 %
5	,,	0.8578	97.9	1.3
23	,,	0.8256	94.2	5.0
30	,,	0.8175	93.3	5.9
39	,,	0.8100	92.5	6.7
54	0.8798	0.7912	89.9	9.3
Glutamic acid at 35°C.				
6 hrs.	0.8896 gr.	0.8768 gr.	98.6%	1.4%
12	0.8932	0.8806	98.6	1.4
30	0.8861	0.8710	98.3	1.7
36	0.8896	0.8680	97.6	2.4
60	0.8896	0.8616	96.9	3.2

Table II.

Time	Total-N. in 100c.c.	NH_2-N (increased) in 100 c.c.	Pyrrolidone-carboxylic acid changed.
Pyrrolidone-carboxylic acid at 50°C.			
3 hrs.	1.052 gr.	0.0558 gr.	5.3%
21	,,	0.3486	33.1
27	,,	0.3828	36.4
35	1.045	0.4632	44.3
54	,,	0.5645	54.4
60	1.055	0.5814	55.1
Pyrrolidone-carboxylic acid at 35°C.			
6 hrs.	1.029 gr.	0.0114 gr.	1.1%
9	1.030	0.0378	3.7
15	,,	0.0597	5.8
33	,,	0.1105	10.8
39	,,	0.1323	12.8
63	,,	0.1860	18.1

Rates of Oxidation of Glutamic Acid and Pyrrolidone-Carboxylic Acid. A solution containing 14.714 gr. of glutamic acid in 280 c.c. of N.-sulphuric acid and a solution containing 12.921 gr. in 280 c.c. of N.-sulphuric acid were electrolysed in undivided cells which were connected in series under the following conditions: The electrodes are of equal size (4×5 cm.), anode: lead peroxide sheet, cathode: lead sheet, C.D.: 2 amp./dm^2., temperature: 19–20°C.

The process of investigation: In the course of electrolysis at every definite consumption of electricity, 10 c.c. of the solution were drawn out and the total ammonia-N (NaOH) in it was determined and its percentage to total-N was calculated, which denotes the percentage of amino-acid oxidised. The results of this experiment are shown in Table III.

Table III.

Faradays per mol.	Oxidised solution.	Glutamic acid oxidised N. in 10 c.c.	Pyrrolidone-carboxylic acid N. in 10 c.c.
1.00 F	0.100 mol 280 c.c.	Total-N, 47.80 mg. NH_3-N 6.68 14.0%	47.80 mg. 16.72 35.0%
2.07 F	0.093 mol 260 c.c.	Total-N, 47.40 mg. NH_3-N 12.90 27.4%	48.50 mg. 31.90 65.8%
4.07 F	0.0858 mol 240 c.c.	Total-N, 46.45 mg. NH_3-N 22.60 48.6%	48.80 mg. 45.40 93.0%
6.24 F	0.0786 mol 220 c.c.	Total-N, 45.70 mg. NH_3-N 31.10 68.1%	48.80 mg. 48.00 98.4%

Electrolytic Oxidation of Pyrrolidone-Carboxylic Acid. Separation of Oxidation Products. 6.45 Gr. of pyrrolidone-carboxylic acid was dissolved in 120 c.c. of N.-sulphuric acid and electrolysed with a lead peroxide anode and a lead cathode with an anodal current density of 2 amp./dm^2. The amount of electricity was 7.97 amp. hours, and the temperature was 23–28°C.

The nitrogen distribution of the oxidised solution was as follows:

Total-N	100.0%
Ammonia-N (MgO)	15.7
Ammonia-N (NaOH)	80.6

The solution was neutralised with barium hydroxide, and the precipitated barium sulphate was filtered off. The filtrate was evaporated to dryness under reduced pressure, and extracted with alcohol (A). The mass insoluble in alcohol was treated with acetone (B), after acidifying with hydrochloric acid. From (B) a small quantity of succinic acid, m.p. 181°C., was obtained.

On distilling off alcohol from (A), crystals were obtained which were found to be almost pure succinimide (3.2 gr.).

Similarly 6.45 gr. of pyrrolidone-carboxylic acid was oxidised with a current quantity of 8.0 amp. hours (at 24°C.). The oxidised solution was

neutralised with potassium hydroxide, evaporated to dryness under reduced pressure, and extracted with absolute alcohol. The crystals formed after removal of alcohol, were recrystallised from acetone in colourless crystals (3.4 gr.), m.p. 126°C., alone or when mixed with a pure sample of succinimide (Kahlbaum).

Anal.: Subst. = 0.1114, 0.0919; CO_2 = 0.1980, 0.1641; H_2O = 0.0489, 0.0406 gr.
Subst. = 0.2626; NH_3 = 26.56 c.c. of N./10–H_2SO_4.
Found: C = 48.49, 48.68; H = 4.89, 4.94; N = 14.17%.
Calc. for $C_4H_5O_2N$ (succinimide): C = 48.48; H = 5.02; N = 14.15%.

From the residue, after separating succinimide, succinic acid, m.p. 183°C., was obtained.

Oxidation of Glutamic Acid. A solution consisting of 530 c.c. of N.-sulphuric acid and 50 gr. of glutamic acid was electrolysed with a lead peroxide anode, the amount of electricity applied being 32.8 amp. hours. The ammonia-N (NaOH) of the oxidised solution amounts to 12.3% of total nitrogen.

The solution was neutralised with barium carbonate, and filtered. The filtrate was evaporated to dryness, and extracted with acetone containing a little hydrochloric acid. The residue, after removal of acetone, was recrystallised from acetone and alcohol, succinamic acid being obtained (0.5 gr.) in colourless crystals, m.p. 154–155°C.

Anal.: Subst. = 0.1155; NH_3 = 10.10 c.c. of N./10–H_2SO_4.
Found: N = 12.26%.
Calc. for $C_4H_7O_3N$ (succinamic acid): N = 11.94%.

The above attempt to obtain succinimide from the electrolysate of glutamic acid by the similar treatments as in the case of pyrrolidonecarboxylic acid was a failure, while a small amount of succinamic acid was obtained. This fact is explained as follows. A small amount of succinimide, perhaps once formed in the solution, may have been completely hydrolysed to succinamic acid during electrolysis and various treatments for its isolation.

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A NEW DITERPENE—γ-CAMPHORENE.

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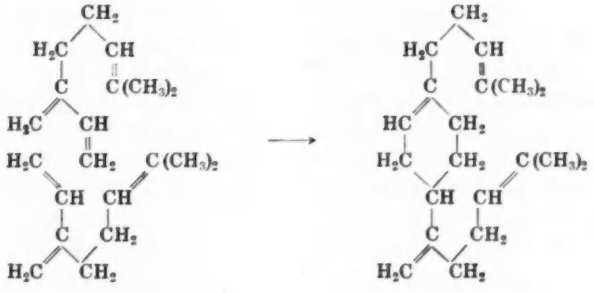
Received April 5th, 1933. Published May 28th, 1933.

It was F. W. Semmler and Irene Rosenberg[1] who first liberated the diterpenes α- and β-camphorenes out of the high-boiling fraction of camphor oil, and shortly afterwards these were synthetically prepared by Semmler and K. G. Jonas[2] from the aliphatic terpene myrcene by the action of heat and pressure on it. These diterpenes are regarded as monocyclic as they give tetrahydrochlorides as well as octahydro-compounds, and also as their molecular refractions accord with $C_{20}H_{32}$ $\models_4$, but, as to their constitutional formulae, nothing exact is yet known. Only, L. Ruzicka and M. Stoll[3] have suggested the following one for α-camphorene from considerations of its mode of formation from myrcene and of the oxidative destruction of its octahydro-derivative, taking into account that myrcene which represents isopentenyl-isoprene, ought to undergo an analogous polymerization as isoprene does to form dipentene, but this deduction is not rigorous enough.

One of the present authors (K), during his former experiments with Formosa lemongrass oil, proceeded with his terpene fraction exactly as described by Semmler and Jonas, in order to identify myrcene in it, and it was that his crude α-camphorene tetrahydrochloride appeared under the microscope to be a conglomerated mixture of two distinctly differently shaped crystals—the one representing thin hexagon plates, the other tiny needle-shaped prisms. Furthermore, he observed that pure α-camphorene tetrahydrochloride corresponding to the descriptions of Semmler and others could only be attained after several consecutive recrystallizations of this crude product from hot ethyl alcohol, and that at the pure stage, no more needle-like crystals were to be seen under the microscope except in very small amount. With a view to clear up the nature of this prismatic contamination, the present authors recently resumed the study in this field of research, and, after a series of experiments, at last arrived at the conclusion that among the diterpenes resulting from the polymerization of myrcene, there existed a hitherto unknown diterpene which leads to a new hydrochloride melting at 96–98°C., and which crystallizes

(1) *Ber.* **46** (1913), 768.
(2) *Ber.* **46** (1913), 1566; **47** (1914), 2077.
(3) *Helv. Chim. Acta*, **7** (1924), 271.

Isoprene. Dipentene.



Myrcene α-Camphorene

in prismatic needles. The reason why this diterpene slipped the elaborate hands of such renowned chemist as Semmler, is because this hydrochloride is readily decomposed by hot alcohol never to crystallize again. To this new diterpene, the authors propose the name "*γ-camphorene.*"

γ-Camphorene has almost the same physical properties as the α-modification as can be seen from the following data:—

	α-Camphorene	γ-Camphorene
b. p. / 4.5 mm.	178°	176–178°
d	$0.8864_{(21/4)}$	$0.8875_{(19/4)}$
n_D	$1.4998_{(21)}$	$1.5030_{(19)}$
M. R.	90.7	90.6
Do. calc. as $C_{20}H_{32}$	90.48	

γ-Camphorene tetrahydrochloride is readily soluble in cold petroleum ether while α-camphorene tetrahydrochloride is not, and thus the two can easily be separated from each other. The γ-hydrochloride can be recrystallized from warm petroleum ether without decomposition, and the purified crystals melt at 96–98°C. The γ-hydrochloride, on treatment

with alcoholic potash, regenerates γ-camphorene and this regenerated hydrocarbon, on being acted on by dry hydrogen chloride gas, redeposits the needle-crystalline γ-camphorene tetrahydrochloride only, and no more plate crystals of α-camphorene tetrahydrochloride with it. By the action of hydrogen bromide gas, α-camphorene gives a tetrahydrobromide melting at 133–134°C., while γ-camphorene yields a corresponding hydrobromide melting at 111–114°C.

Experimental.

Myrcene. The starting material myrcene was obtained partly from Formosa lemongrass oil and partly from linalool. That from the former was prepared by carefully fractionating the crude oil dried over anhydrous sodium sulphate under 50 mm. pressure, and then shaking the terpene fraction thus obtained with 40% sodium bisulphite solution, and finally distilling the product over metallic sodium. The other portion from linalool was obtained in the following way: To pure linalool kept in a flask and heated to 100–120°C., iodine in form of fine powder was slowly added, when a violent reaction took place. The mass was distilled under 100 mm. pressure, and the process was repeated with the distillate until all linalool decomposed. Then the product was distilled over metallic sodium and was rectified under 12 mm. pressure. Myrcene thus obtained showed the following physical properties:—

b. p. / 12 mm.	55°C.
d	$0.8030_{(19/4)}$
n_D	1.4680

Polymerization of Myrcene. Myrcene was heated in a sealed tube in a bomb furnace up to 240–260°C. for four hours. After cooling, the content of the tube was fractionated under 6 mm. pressure, when about 30% came over between 51° and 55°C., which turned out to be an isomerized monocyclic terpene with some unchanged myrcene, the properties of which are as follows:

b. p.	51—55°C. / 6 mm.
d	$0.8420_{(22/4)}$
n_D	$1.4680_{(22)}$
M. R.	44.9
Do. calc. as $C_{10}H_{16}$ $\equiv_2$	44.90

The next 44% distilled between 162° and 176°C., and showed the following constant:

b. p.	162–176°C. / 6 mm.
d	$0.9203(^{24}/_{4})$
n_D	$1.4960(_{24})$
M. R.	88.3
Do. calc as $C_{20}H_{32}$ $\|=_4$	90.48

These constants are in accordance with Semmler's data for crude camphorene.

α- and γ-Camphorene Tetrahydrochloride. The crude camphorene thus obtained was dissolved in its own volume of absolute ether and was saturated with dry hydrogen chloride gas and stood overnight in cold. The gruel-like mass of crystals thus produced was freed from the adhering liquid on porous porcelain and then it was boiled with light petroleum ether and filtered while warm. From the filtrate there precipitated on cooling the needle-like crystals of γ-camphorene tetrahydrochloride m.p. 96–98°C. The filtered crystals were washed with warm petroleum ether several times, dried, and then recrystallized from hot alcohol. The crop of crystals so obtained, on inspection under the microscope contained no more needle-shaped crystals of γ-camphorene tetrahydrochloride, and melted at 129–131°C., representing pure α-camphorene tetrahydrochloride.

Anal.: Crystals (m.p. 96–98°C.) = 2.730; AgCl = 3.762 mg.
Found: Cl = 34.17%. Calc. for $C_{20}H_{36}Cl_4$: Cl = 33.91%.
Crystals (m.p. 129°–131°C.) = 3.386; AgCl = 4.480 mg.
Found: Cl = 33.50%. Calc. for $C_{20}H_{36}Cl_4$: Cl = 33.91%.

Regeneration of α- and γ-Camphorenes. Ten grams each of α- and γ-camphorene tetrahydrochlorides were separately dissolved in an excess of 10% alcoholic potash, boiled under reflux for seven hours on the water-bath, saturated with carbon dioxide, filtered from depositing solid matters, alcohol distilled off, and finally the residual substance was distilled in vacuo over metallic sodium. The yield of the ultimate product in each case amounted, in the case of α-camphorene to about 98%, and in that of γ-camphorene to about 75% of the theory, and each exhibited such properties as given in the preceeding page. These, on treatment with hydrogen chloride, gave the respective hydrochlorides only, and not the mixture of the two hydrochlorides.

α- and γ-Camphorene Tetrahydrobromides. On treatment of these two diterpenes with hydrogen bromide gas, tetrabromides resulted. From α-camphorene lustrous scaly crystals crystallized out which after purification melted at 133–134°C., while from γ-camphorene, prismatic needle

crystals separated out with m.p. 111–114°C. Both could be recrystallized from petroleum ether, but the γ-modification was more soluble than the α-hydrobromide, and moreover, it exhibited a remarkable tendency to decompose on dissolving in hot alcohol.

Anal.: α-Hydrobromide = 5.910; AgBr = 7.500 mg.
Found: Br = 53.96%. Calc. for $C_{20}H_{36}Br_4$: Br = 53.66%.
γ-Hydrobromide = 4.937; AgBr = 6.180 mg.
Found: Br = 53.34%. Calc. for $C_{20}H_{36}Br_4$: Br = 53.66%.

Camphorene Octabromide. By the action of bromine, α- and γ-camphorenes give an undefined crystalline octabromide, seemingly identical. For this purpose, an ethereal solution of each hydrocarbon was treated by an excess of bromine, also in the same medium. The reaction mass was stood still in an ice chamber overnight, excess of bromine and ether expelled in vacuo, the residue which at first assumed a syrupy consistency, gradually solidified on keeping. Then, it was powdered, redissolved in ether, a little methyl alcohol added thereto, filtered, and evaporated in cold. Thus, from both hydrocarbons, a white crystalline powder was obtained which melted between 70° and 80°C., showing no lowering of the melting point by mixing with each other. Other methods of purification were tried to attain to a more definite melting point but in vain.

Anal.: Substance from α-camphorene = 6.105; AgBr = 10.026 mg.
Substance from γ-camphorene = 4.237; AgBr = 6.871 mg.
Found: Br = 69.91; 69.01%. Calc. for $C_{20}H_{32}Br_8$: Br = 70.17%.

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GLUCOSULFATASE. V.

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Received April 18th, 1933. Published May 28th, 1933.

Glucosulfatase[1] is regarded as an enzyme which hydrolyses glucose-monosulphate.[2] Some of its properties, however, are still ambiguous. For instance, we do not know if: (1) it is simply a hydrolysing enzyme, (2) it can be obtained in pure state and (3) it can be obviously distin-

(1) T. Soda and C. Hattori, this Bulletin, **6** (1931), 258.
(2) T. Soda, *Biochem. Z.* **135** (1923), 623; this Bulletin, **8** (1933), 37.

guished from phenosulfatase. In the present paper we have chiefly dealt with these questions.

As experimental materials, we used at first the internal organs of *viviparus japonicus* (*tanishi*)[3] and afterwards those of *charonia lampas* (*boshu-bora*),[4] the liver of which is found to be the best material.[5]

Experiment A. I. The effect of the time of autolysis and the effect of $CaCO_3$ as a neutralising agent.

Enzyme solution. (a) Ten grams of the internal organs of *viviparus japonicus* dried with acetone were autolysed for 3 days in 60 c.c. of water at 35°C. in the presence of a little chloroform (we used it always as an antiseptic during the autolysis). The enzyme solution was then separated with a centrifuge. (b) Forty grams of the enzyme material were autolysed for 3 hours in 240 c.c. of water and the enzyme solution was obtained in the same way as in (a). Substrate solution. Solution of potassium glucose-monosulphate, 5 c.c. of which gave 0.0999 gr. $BaSO_4$.

The compositions of the reaction mixture and of the controls were as follows:

Reaction mixture:		Enzyme solution	50 c.c.
		Substrate solution	50
		Chloroform	2
Controls:	(1)	Enzyme solution	50 c.c.
		Chloroform	1
		With or without $CaCO_3$	
	(2)	Substrate solution	50 c.c.
		Chloroform	1
		With or without $CaCO_3$	

These solutions were kept at room temperature (ca. 20°C.) and the controls (1) and (2) were mixed just before it was put to analysis.

The method of estimation: 10 c.c. of the reaction mixture (or 5 c.c. of each control) were mixed with 10 c.c. of 20% trichloroacetic acid and the protein-precipitate was separated with a centrifuge. Fifteen c.c. of the clear solution were mixed then with 10 c.c. of 5% $BaCl_2$ solution and 15 c.c. of 5% KOH solution. It was immediately separated again from the precipitate in the same way as before. Now 30 c.c. of the clear solution were hydrolysed by HCl and thus the substrate, which had not been previously decomposed by the enzyme, was determined in the form of $BaSO_4$ by the ordinary gravimetric method. Result of experiments:

(3) T. Soda and C. Hattori, *J. Chem. Soc. Japan*, **54** (1933), 59.
(4) „ „ , this Bulletin, **8** (1933), 65.
(5) „ „ , *J. Chem. Soc. Japan*, **54** (1933), 377.

Table 1.

	Enzyme	$CaCO_3$	1 day	5 days	9 days	13 days
No. 1.	a	with	29.1%	44.7%	44.7%	46.0%
No. 2.	b	with	5.6	22.9	37.3	43.6
No. 3.	b	without	9.0	25.0	26.2	26.2
Control	b	with	—	—	—	0.4
,,	b	without	—	—	—	0.2

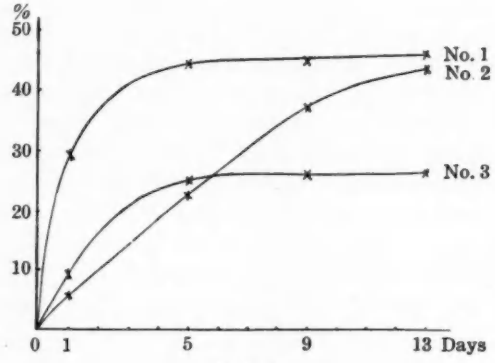

Fig. 1.

The percentage in this table expresses the rate of decomposition of the substrate. From these experiments we see that the enzyme solution (a) (autolysed for 3 days) was in the beginning much more active than that of (b) (autolysed for 3 hours). And we see from No. 2. and No. 3. that $CaCO_3$ is available for this kind of experiment as a neutralising agent.

II. The evidence of simply hydrolytic nature of the enzyme.

In order to ascertain that this enzyme actually hydrolyses glucose-monosulphate in the following way:

$$C_6H_{11}O_5 \cdot O \cdot SO_3K + H_2O = C_6H_{12}O_6 + KHSO_4$$

and has no other action than this, we tried to estimate the quantity of glucose produced by glucosulfatase. For this purpose the glucose thus produced was fermented by yeast and the volume of CO_2 obtained was approximately determined with the apparatus of Lunge and Rittener.[6]

(6) *Z. angew. Chem.*, **19** (1906), 1849.

Now, if we assume that the hydrolysis by the enzyme and the fermentation by yeast have been complete, 10 c.c. of the reaction mixture in the above experiment (I) would give at normal state

$$2 \times 22412 \times \frac{0.0999}{BaSO_4} = \frac{2 \times 22412 \times 0.0999}{233.5} = 19.18 \text{ c.c. of } CO_2$$

according to the equation of ordinary fermentation:

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

As for the blank test, we have ascertained that the mixture of 5 gr. yeast, 5 c.c. enzyme solution and 45 c.c. of water, on standing, evolved no CO_2. From the mixture of 5 gr. yeast, 10 c.c. of sample No. 2. in the forgoing article (hydrolysed for 9 days) and 40 c.c. water evolved 7.83 c.c. CO_2, at the atm. pressure (P) = 757.8 mm., the temperature (t) = 22.3°C. and vapour tension of water (p) = 20.3 mm. The volume of CO_2 at N.S. (V) has been calculated from the observed volume (v) by the following formula:

$$V = v \frac{273.1}{273.1 + t} \cdot \frac{760}{P - 0.8p} \quad (7)$$

From the above data we have $V = 7.06$ c.c. Therefore the percentage of glucose hydrolysed by the enzyme can be calculated as follows:

$$7.06/19.18 \times 100 = 36.8\% .$$

The percentage obtained from the determination of SO_4 = 37.3%, as is shown in Table 1. This coincidence of both percentages could be regarded as the proof that glucosulfatase is simply a hydrolysing enzyme. After 20 days the same experiment as above was repeated. The blank test, however, gave this time 2.29 c.c. of CO_2 therefore this amount was subtracted from the volume 16.65 c.c. of CO_2 evolved by fermentation, and we obtain $v = 14.36$ c.c. at $t = 25.5°$, $P = 756.2$ mm., $p = 24.5$ mm. Consequently $V = 12.73$ c.c., and the enzymic hydrolysis expressed in percentage is $12.73/19.18 \times 100 = 66.4\%$. The corresponding percentage calculated from the determination of SO_4 is 64.2%.

Experiment B. Adsorption and purification of the enzyme.

The dry enzyme preparations were made from *charonia lampas* in the same way as in Experiment A. The substrate solution was either

(7) The factor "0.8" in this formula is to reduce the vapour pressure of pure water to that of saturated NaCl-solution or of KOH-solution (1:2).

sodium or barium salt of glucose-monosulphate of 0.0709 N.[8] The experiments were always carried out at 30.0°C. in a thermostat.

I. Adsorption by kaolin.

Enzyme solution: No. 1. 20 Gr. of the dry enzyme preparation were autolysed for a day in 100 c.c. of water at 32°C. and the clear enzyme solution was separated with a centrifuge. No. 2. 8 Gr. of kaolin were added to 25 c.c. of the above enzyme solution (No. 1), and it was clarified again as above and made up to 25 c.c. by adding water to it (kaolin here used was that of Merck heated for about 2 hours at 200°C.).

Substrate solution:		Solution of sodium glucose-monosulphate.	
Reaction mixture:		Substrate solution	30 c.c.
		Enzyme solution	20
		Water	10
		Chloroform and $CaCO_3$	
Controls:	(1)	Substrate solution	30 c.c.
		Chloroform and $CaCO_3$	
	(2)	Enzyme solution	20 c.c.
		Water	10
		Chloroform and $CaCO_3$	

The experiment procedure: same as is described in Experiment A. Result of experiments:

Enzyme	1 day	2 days
No. 1.	13.4%	20.1%
No. 2.	1.3	1.5
Control (No. 1.)	—	3.6

We see that the enzyme was almost perfectly adsorbed by kaolin. The 3.6% hydrolysis observed in the control is probably due to the presence of some unknown ethereal sulphate in the enzyme preparation.

Two c.c. of both enzyme solutions were dried separately in vacuum over $CaCl_2$. The weight of No. 1. and No. 2. after being dried were 0.0918 gr. and 0.0324 gr. respectively. From these data we have attempted to calculate the concentration of glucosulfatase. Assuming that the reaction proceeds as monomolecular (on this point we shall deal later on) the velocity constant (k) was calculated as follows:

$$k = \frac{1}{t} \log \frac{a}{a-x} = \log \frac{100}{100-13.4} = 0.0625 \quad \text{(Enzyme No. 1.)}$$

(8) Concentration of substate solutions is always of the same normality if not otherwise stated.

As 20 c.c. of enzyme solution were diluted with 10 c.c. of water, the actual dried weight of 2 c.c. of the enzyme solution should be

$$m = 0.0918 \times 2/3 = 0.0612 \text{ gr.}$$

If we express the concentration of our glucosulfatase as "G-Sulf." (Glucosulfatase-fähigkeit), so the concentration of Enzyme No. 1. is: G-Sulf $= k/m = 1.0$; Enzyme No. 2.: G-Sulf $= 0.3$.

II. Adsorption experiments with $BaSO_4$ and aluminium hydroxide gel.[9]

Enzyme solution: No. 1. 15 Gr. of the dry enzyme preparation were autolysed for 2 days in 80 c.c. of water at 32°C. and the enzyme solution was separated with a centrifuge.

No. 2. Ten c.c. of aluminium hydroxide gel were added to 25 c.c. of the enzyme solution No. 1, and the clear enzyme solution No. 2 was separated by means of a centrifuge.

No. 3. Five c.c. of 10% $BaCl_2$ solution were added to 25 c.c. of the enzyme solution No. 1 which contained some inorganic sulphate. Then the enzyme solution No. 3 was separated from the precipitate (which contained proteins and $BaSO_4$) with a centrifuge.

Substrate solution:	Solution of sodium glucose-monosulphate.	
Reaction mixture:	Substrate solution	20 c.c.
	Enzyme solution	20
	Chloroform and $CaCO_3$	

Result of experiments:

Enzyme	Rate of hydrolysis in 2 days	Weight of 2 c.c. enzyme solution dried	Concentration of enzyme (G-Sulf)
No. 1.	24.4%	0.0595 gr.	1.0
No. 2.	22.7	0.0398	1.4
No. 3.	75.7	—	—

From the above result, it is obvious that aluminium hydroxide gel adsorbs scarcely the enzyme, but it removes the impurities fairly well.

(9) Preparation of aluminium hydroxide gel: saturated solution of $AlCl_3.6H_2O$ (20 gr.) was added to 500 c.c. of warm ammonium hydroxide solution (5%). Then the solution was boiled for about 7 hours keeping its volume as constant as possible. It was then dialysed for several weeks. Two c.c. of the gel thus obtained, after being dried in vacuum over $CaCl_2$, weighed 0.0138 gr., and after ignition it weighed 0.0091 gr. as Al_2O_3.

The most remarkable fact is the increase of activity by the treatment with $BaCl_2$ (Enzyme No. 3). We have, therefore, studied the purification of the enzyme by applying these two methods and found very effective as is described in the next article.

III. Purification of the enzyme with $BaCl_2$ and $Al(OH)_3$ gel.

Enzyme solution: 10 Gr. of the dry enzyme preparation were autolysed for 2 days in 50 c.c. of water at 32°C. Then it was separated with a centrifuge. Ten c.c. of 10% $BaCl_2$ solution were added to it and again separated as before. The solution was then treated with 5 c.c. of $Al(OH)_3$ gel and subsequently centrifuged. A quite clear enzyme solution was thus obtained.

Substrate solution:	Solution of barium glucose-monosulphate with an excess of $BaCl_2$.	
Reaction mixture:	Substrate solution	20 c.c.
	Enzyme solution	20
	Chloroform and $CaCO_3$	

The method of estimation: As the enzyme solution thus obtained was almost free from proteins, the treatment with trichloroacetic acid was omitted and the procedure was simplified as follows:

Ten c.c. of the filtered reaction mixture were decomposed by HCl and the amount of substrate was determined as usual in the form of $BaSO_4$. Result of the experiment: The amount of hydrolysis in a day = 82.0%, $k = 0.745$; the weight of 2 c.c. enzyme solution dried = ca. 0.11 gr. (in which some excess of $BaCl_2$ is contained; therefore the original weight must be less than this). From these data we have: G-Sulf. = 7.

IV. Adsorption experiment with charcoal.

Enzyme solution: 30 Gr. of the dried liver were autolysed for 2 days in 120 c.c. water at 32°C. It was separated with a centrifuge and 4 c.c. of Na_2SO_4 solution (saturated at 15°C.) and 12 c.c. of 10% solution of $BaCl_2$ were successively added to it. Then it was centrifuged again. Ten c.c. of $Al(OH)_3$ gel were added to it. After centrifuging off the gel, the solution was treated with 5 gr. of charcoal and subsequently filtered. This enzyme solution was perfectly clear.

Substrate solution:	Solution of barium glucose-monosulphate with an excess of $BaCl_2$.	
Reaction mixture:	Substrate solution	30 c.c.
	Enzyme solution	30
	Chloroform and $CaCO_3$	

Result of the experiment:

The rate of hydrolysis in a day = 50.1 %
The velocity constant k = 0.302
The weight of 2 c.c. enzyme solution dried = 0.0378 gr.
,, ,, ,, ash ($BaCl_2$, NaCl etc.) = 0.0051
,, ,, ,, enzyme, proteins and unknown org. substances = 0.0327

Hence G-Sulf = 0.302 / 0.0327 = 9.5

Fifty c.c. of this enzyme solution were dialysed for 2 days through a collodion membrane and was precipitated in acetone and dried over $CaCl_2$ in vacuum. The yield was ca. 0.2 gr. It was readily and perfectly soluble in water. Fifteen c.c. of the substrate solution was hydrolysed by 0.0644 gr. of this preparation dissolved in 15 c.c. of water. The hydrolysis proceeded 25% in a day. Hence

$$\text{G-Sulf} = \frac{1}{0.0644 \times 2/15} \log \frac{100}{100-25.0} = 11.6$$

We have thus obtained a solid preparation of glucosulfatase which is 11.6 times more concentrated than the original enzyme.

Experiment C. I. Effects of glucose and galactose upon enzyme action.

Enzyme solution: 20 Gr. of internal organs of *viviparus japonicus* dried with acetone were autolysed for a week in 100 c.c. of water at room temperature (20–28°C.) and centrifuged.

Substrate solution: Solution of potassium glucose-monosulphate, 5 c.c. of which gave 0.0999 gr. $BaSO_4$.

Reaction mixtures: No. 1. Enzyme solution 20 c.c.
Water 10
Substrate solution 30
Chloroform and $CaCO_3$

No. 2. 10 c.c. of glucose solution (2.446 gr. glucose in 100 c.c.) instead of water in No. 1.

No. 3. 10 c.c. of galactose solution (2.450 gr. in 100 c.c.) instead of water in No. 1.

Results of experiment:

	1 day	2 days	5 days	10 days
No. 1.	3.0%	7.3%	16.3%	23.1%
No. 2.	1.0	2.7	10.7	13.6
No. 3.	1.0	2.8	10.8	14.5

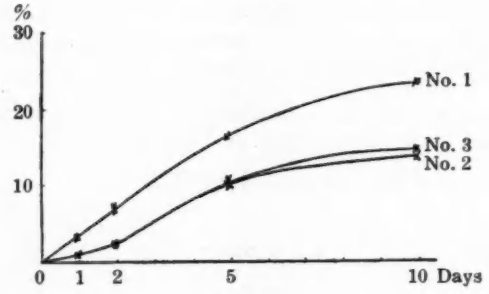

Fig. 2.

According to these results, glucose and galactose inhibit the action of glucosulfatase and indeed in about same degree.

II. Effects of inorganic sulphate and phosphate and magnesium carbonate.

The inhibiting action of phosphate upon phosphatase has been studied by many biochemists. Recently E. Jacobsen[10] studied also this problem and he explained the mechanism of this inhibition by the affinity between the phosphate and the enzyme. Therefore it would be interesting to study the action of sulphate ion upon glucosulfatase in the analogous way.

According to C. Hommerberg,[11] phosphate has also inhibiting action upon phenosulfatase and therefore we must remove phosphate ion, if we want to obtain very active phenosulfatase.

The effect of magnesium salt, an activator of phosphatase,[12] upon phenosulfatase has also been studied. Neuberg and Linhardt[13] tried to use $MgCO_3$ instead of $CaCO_3$ as a neutralising agent, whereas C. Hommerberg[14] used $MgCl_2$ in order to examine the effect of Mg-ion. According to them, Mg-ion has always a remarkable inhibiting action upon phenosulfatase. It would be, therefore, also significant to examine the actions of phosphate and $MgCO_3$ upon glucosulfatase.

Enzyme solution: 20 Gr. of the dry liver of *charonia lampas* were autolysed for 2 days in 80 c.c. of water at 32°C. Crude enzyme solution was separated with a centrifuge and 4 c.c. of Na_2SO_4 solution and 12 c.c.

(10) *Biochem. Z.*, **249** (1932), 21.
(11) *Z. physiol. Chem.*, **200** (1931), 79.
(12) Erdtman, *Z. physiol. Chem.*, **177** (1928), 211.
(13) *Biochem. Z.*, **142** (1923), 192.
(14) *Z. physiol. Chem.*, **200** (1931), 80.

of 10% $BaCl_2$ solution were successively added. The precipitate was centrifuged off and the solution was shaken with 2 gr. of charcoal, thereafter it was filtered and dialysed.

Substrate solution:	Solution of sodium glucose-monosulphate.	
Reaction mixtures:	Substrate solution	30 c.c.
	Enzyme solution	10
	Buffer solution (acetate buffer, pH: 7)	10
	Water	5
	1. Water	5
	or (a) Sulphate solution: 12% Na_2SO_4	
	or (b) Phosphate solution: $NaH_2PO_4+Na_2HPO_4$, pH = 7; 6% as P_2O_5	
	2. Water and $CaCO_3$ or Water and $MgCO_3$	5

Method of estimation: Ten c.c. of the reaction mixture were treated with 6 c.c. of 10% $BaCl_2$ solution and 4 c.c. of 1% KOH solution and it was then filtered. Fifteen c.c. of the filtrate were put to analysis as already described (Experiment B, III).

Results of experiments:

		The rate of hydrolysis in 2 days
1.	Water	27.6%
	Sulphate (a)	13.6
	Phosphate (b)	0.0
2.	$CaCO_3$	16.6
	$MgCO_3$	9.2

The inhibiting action of phosphate is emormously strong; that of sulphate and $MgCO_3$ is also evident.

Experiment D. On glucosulfatase and phenosulfatase in the liver of *charonia lampas*.

I. Comparison of the activities of glucosulfatase and phenosulfatase.

Enzyme solution: 10 Gr. of the liver dried with acetone were autolysed for 2 days in 50 c.c. of water at 32°C., and from this the enzyme solution was prepared after the procedure described in Experiment B, III.

Substrate solutions: No. 1. solution of barium glucose-monosulphate with an excess of $BaCl_2$. No. 2. solution of sodium phenolsulphate with an excess of $BaCl_2$. Both solutions had the same normality. (0.0709 N).

Reaction mixture:	Enzyme solution	20 c.c.
	Substrate solution	20 c.c.
	Chloroform and $CaCO_3$	

Result of experiment:

Substrate	The rate of hydrolysis in a day
No. 1. (Gl)	78.8%
No. 2. (Ph)	75.6

Neuberg and Simon[15] as well as Hommerberg[16] tried to prepare phenosulfatase from some mammalian organs. The former could extract the enzyme but with only weak activity (8% hydrolysis in several days), while the latter did not succeed to prepare it. Phenosulfatase obtained from the liver of *charonia lampas* has probably the strongest activity ever extracted from animal organs (almost comparable to that of Taka-diastase).

II. Adsorption of glucosulfatase and phenosulfatase by kaolin.

Enzyme solution: No. 1. 30 Gr. of the dried liver were autolysed for 2 days in 120 c.c. of water 32°C. and further treated as in Experiment B, III (10% $BaCl_2$: 20 c.c., $Al(OH)_3$: 20 c.c.).

No. 2. 15 Gr. of kaolin were added to 60 c.c. of the enzyme solution (No. 1.) and it was separated with a centrifuge. The clear solution was diluted with water to 60 c.c.

No. 3. The kaolin which adsorbed the enzyme was washed with water.

Substrate solutions: No. 1. solution of barium glucose-monosulphate with an excess of $BaCl_2$. No. 2. solution of sodium phenolsulphate with an excess of $BaCl_2$.

Reaction mixtures:

No. of enzyme	No. 1.	No. 2.	No. 3.
Substrate solution	70 c.c.	20 c.c.	20 c.c.
Enzyme solution	70	20	20 c.c. water + 6 gr. kaolin with enzyme

Chloroform and $CaCO_3$ were added.

Result of experiments:

(15) *Biochem. Z.*, **156**(1925), 372.
(16) *Z. physiol. Chem.*, **200**(1931), 69.

Subst.		Enzyme	3 hours	6 hours	21 hours	58 hours
(a)	No. 1.	No. 1.	17.6%	33.1%	73.9%	75.1%
(b)	No. 1.	No. 2.	—	—	—	1.6
	No. 2.	,,	—	—	—	2.5
(c)	No. 1.	No. 3.	—	—	—	29.5
	No. 2.	,,	—	—	—	49.3

The reaction velocity constants calculated as monomolecular reaction from data of experiment (a) are:

Hours	3	6	21	58
k	0.028	0.029	0.028	0.012

Therefore the reaction is monomolecular at least for first 20 hours. Both enzymes are adsorbed well by kaolin with about the same ratio as in the solution. It seems hopeless to separate these two enzymes by the adsorption method such as described above.

III. Evidence that the phenosulfatase and the glucosulfatase are not one and the same enzyme.

We have seen that the glucosulfatase always hydrolyses also phenolsulphate. We do not know, however, whether the glucosulfatase can by itself hydrolyse phenolsulphate or it is always mixed with the phenosulfatase which had been studied by Neuberg and his co-workers. In this experiment, we attempted to distinguish these two enzymes by their different stability against weak alkali and also by the inhibiting action of glucose upon both enzymes.

Enzyme solution: The enzyme solution (A) was prepared in the same way as described in Experiment C, II, but without dialysis.

No. 1. Forty c.c. of the enzyme solution (A) were diluted with 4 c.c. of water. No. 2. To 20 c.c. of the enzyme solution (A) 1 c.c. of 2 N. ammoniumhydroxide solution was added and kept for 16 hours. Then it was neutralised by 2 N. acetic acid.

Substrate solution: solutions of barium glucose-monosulphate and sodium phenolsulphate both with an excess of $BaCl_2$.

Reaction mixture:	Substrate solution	15 c.c.
	Enzyme solution	10 c.c.
	Water or glucose solution (10%)	5 c.c.
	Chloroform and $CaCO_3$	

Results of experiment:

Substrate	Enzyme	Water or glucose soln.	The rate of hydrolysis in a day
Glucose-sulphate	No. 1.	water	70.1%
,,	No. 2.	,,	13.6
,,	No. 1.	glucose	61.1
Phenol-sulphate	No. 1.	water	29.0%
,,	No. 2.	,,	36.6
,,	No. 1.	glucose	27.8

The above data show that the action of glucosulfatase is strongly inactivated by weak alkali while that of phenosulfatase is rather activated by it. Moreover, glucose which has remarkable inhibiting action upon glucosulfatase scarcely inhibits the action of phenosulfatase. These facts may serve for the evidence that glucosulfatase and phenosulfatase are not one and the same enzyme.

The difference of activities of glucosulfatase and phenosulfatase in this experiment is very remarkable compared with that observed in Experiment D, I. Charcoal seems to adsorb phenosulfatase.

Summary.

1. Glucosulfatase is a simply hydrolysing enzyme.
2. It is almost perfectly adsorbed by kaolin.
3. It can not be adsorbed by $BaSO_4$, aluminium hydroxide gel and charcoal. These adsorbents can be used for the purification of the enzyme. Thus a perfectly clear enzyme solution is obtainable. A solid preparation of very active glucosulfatase was obtained. Its purity (G-Sulf) is 11.6 while the original value was 1.0.
4. The enzyme action is inhibited by: glucose, galactose, $MgCO_3$, inorganic sulphate and phosphate.
5. Glucosulfatase always contains phenosulfatase but they are not one and the same enzyme.

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ON NIGAKI OIL.

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Received April 18th, 1933. Published May 28th, 1933.

"Nigaki" (literally, "bitter tree"), *Picrasma quassioides Benn.* (*Simarubaceae*), is a shrub or tree grown in hills and fields, and has yellowish-green blossoms from May to July; the fruits are of the size of a pea. The plant has an intense bitter taste, and the bitter substance in the ligneous parts is used as a stomachic, and the decoction sap as an insecticide. The plant is distributed in Japan and China, and also in the Himalaya; in Chôsen (Corea) it is found in almost all mountaineous districts.

No mention of nigaki oil is found in the literature. The oils from the *Simarubaceae* mentioned in the literature have mostly been obtained from tropical plants. It is interesting to note that tariri fat obtained from the genus *Picramnia* belonging to the *Simarubaceae* has been reported to contain a peculiar acid called "tariric acid." The acid melts at 50.5°C., has the formula $C_{18}H_{32}O_2$, and is thus an isomer of linolic acid; but the acid contains a triple bond, its constitutional formula being $CH_3.(CH_2)_{10}.C:C.(CH_2)_4.COOH$, and resembles in this respect to stearolic acid.[1]

In our study of nigaki oil, a particular attention has been directed as to the occurrence of tariric acid in the oil, but with negative result. On the other hand we have confirmed that the oil contains a large proportion of petroselinic acid, an isomer of oleic acid.

Experimental Part.

(1) General Properties of Nigaki Oil. The nigaki fruits used for this experiment were procured from Chûsei-Nandô, Chôsen. They were of a nearly spherical form, and had rumples on the rinds. Most of them had a brown colour, while the others were brownish-yellow or brownish-green. As the fruit shell rather easy to be stripped off, it was not difficult to separate it from the kernel. The kernels were dark yellow and had brown spots. The weight of 50 c.c. of the fruits was 230 gr., and 610 fruits weighed 50 gr. The ratio of the shell to the kernel was about 45 : 55. On analysis the following figures were obtained:

(1) Arnaud, *Bull. Soc. Chim.*, 1892, (3), 7, 233.

	Moisture	Oil
Shell	7.49%	38.36%
Kernel	5.96%	46.57%

From 2240 gr. of the fruits, 660 gr. of the oil were obtained by pressing.

There are three kinds of nigaki oil, viz., obtained (1) from the whole fruit, (2) from the fruit shell, and (3) from the kernel. The nigaki oil mentioned in this paper represents the oil obtained from the whole fruit as mentioned above, but the other two oils have also been prepared and their properties examined.

Nigaki oil was a brownish-yellow, viscous liquid at the room temperature of 26°C., and deposited a large amount of crystalline solid in winter. It had a bitter taste and an unpleasant odour. Its characteristics, etc., were determined as follows:

d_4^{20}	0.9322	Iodine value (Wijs)	76.7
n_D^{20}	1.4712	Reichert-Meissl value	1.21
Acid value	19.0	Acetyl value	28.70
Saponification value	200.5	Unsaponifiable matter	1.48%

On cooling to 0°C., the oil did not solidified in an hour, but at –20°C. it solidified to a salve-like mass, which regained its mobility at 15°C.

The fatty acids had a brownish-yellow colour, and consisted of liquid and solid substances at 26°C. They showed the neutralisation value 207.0 and the iodine value 79.5. By the lead-salt-ether method, 76.7% of liquid and 23.3% (by difference) of solid acids were obtained; they had the following properties:[2]

	Melt. pt.	Neutr. v.	Iodine v.	n_D^{20}
Solid acids	57.5–58°C.	219.6	14.0	—
Liquid acids	—	200.6	93.8	1.4613

On bromination the liquid (unsaturated) acids produced a small amount of precipitate in petroleum ether solution; in ether only a very small amount of precipitate was formed.

(2) Examination of the Composition of the Fatty Acids. The oil was heated with an equal weight of methyl alcohol containing 2.5% of hydrogen chloride on a water bath for about 12 hours. The fractional

(2) This experiment was performed in summer season. At lower temperature (ca. 10°C.) lead petroselinate is sparingly soluble in ether. As petroselinic acid is solid at the ordinary temperature, the designation of "liquid" acids would be incorrect, and is used here only conventionally.

distillation of 500 gr. of the methyl esters thus prepared under 15 mm. pressure gave the following result:

	Yield		Appearance	Saponif. value	Iodine value	n_D^{20}
	gr.	%				
(1) Until 180°	5.9	1.2	Pale yellow liquid	265.8	45.0	1.4420
(2) 180–190°	5.5	1.1	,,	258.2	48.3	1.4444
(3) 190–200°	16.4	3.3	,,	230.1	55.6	1.4469
(4) 200–210°	155.0	31.0	Light orange-yellow liquid	199.8	68.0	1.4493
(5) 210–215°	215.9	43.2	,,	193.5	85.1	1.4519
(6) 215–216°	35.8	7.2	,,	192.4	93.3	1.4540
(7) Residue	ca. 65	ca. 13	Dark brown viscous liquid	—	—	—

The distillation nearly ceased at 216°C. The comparatively large amount of the residue was probably due to somewhat incomplete esterification, and possibly also to the presence of some high boiling compounds in the shell oil.

The above result showed that the chief constituents are the esters of C_{18} acids boiling between 200–216°C. under 15 mm. pressure, which amounted to more than 80%.

On saponifying the fractions (1), (2), and (3), the fatty acids were separated into solid and liquid parts by the lead-salt-ether or lead-salt-alcohol method. From the solid acids palmitic acid was identified. From the fraction (1), an acid of the neutralisation value of 282 was obtained, which appeared to be impure lauric acid. Although not isolated, myristic acid was likely to be present. Of the liquid (unsaturated) acids, the presence of the lower acids (C_{12} to C_{16}) was not ascertained, but those from the fraction (3) consisted of C_{18} acid (probably petroselinic acid).

The fractions (4), (5), and (6) were used for the identification of C_{18} acids. As a preliminary test, a mixture of the corresponding amounts of (5) and (6) was re-fractionated under 15 mm. pressure, and the fatty acids obtained from the fraction boiling at 212–214°C. were treated by the lead-salt-ether method. After the separation of the solid acids (chiefly palmitic), the unsaturated acids were changed into lithium salts and dissolved in 80% alcohol. The salt deposited on cooling was decomposed with hydrochloric acid, and free acid separated. The acid had the neutralisation value 199 and the iodine value 87. As it was liquid in summer season, the presence of tariric acid, a solid of comparatively high melting point, seemed to be excluded. At first we considered the substance to be

ordinary oleic acid, but by the elaidin test, an acid of m.p. 50°C. was obtained. Also the original substance solidified to a crystalline mass at a lower temperature. So we were convinced that the substance consists of a solid octadecenoic acid.

To prepare a larger amount of the acid, the fractions (4)-(6) and the residue (re-esterified) were mixed and fractionated. The fraction 206–212°C. (14–15 mm.) was similarly treated as above, and the crude octadecenoic acid recrystallised from dilute acetone by ice-cooling, and finally the admixed saturated acids were removed as far as possible by means of magnesium acetate. The acid thus purified formed a crystalline mass, melted at 29–29.5°C., and had the neutralisation value 199.7 and the iodine value 86.0 (calc. for $C_{18}H_{34}O_2$, neutr. value 198.8, iodine value 89.9). Judged from the iodine value, it still contained a small proportion of saturated acids. On hydrogenation the acid was changed into stearic acid. By the elaidin reaction, an elaidic acid of m.p. 53°C. was produced. The hydroxy-acid obtained by Hazura's method melted at 121–121.5°C. From these results, the octadecenoic acid appeared to be petroselinic acid.

A small amount of a liquid octadecenoic acid was found to accompany petroselinic acid; it probably consisted of oleic acid.

In the above experiment, the free acids obtained from the filtrate of the lithium salts in alcohol had the iodine value 114. On bromination they gave insoluble precipitates in petroleum-ether and ether solutions; so linolic and linolenic acids were likely to be present. The presence of the latter was highly probable by the melting point (178°C.) of the ether-insoluble bromide.

(3) Confirmation of Petroselinic Acid. Although the octadecenoic acid described above appeared in all probability to be petroselinic acid, its constitution has been confirmed by the oxidation method with potassium permanganate.[3]

20 Gr. of the methyl ester of the acid were dissolved in 200 c.c. of acetone and oxidised with 80 gr. of potassium permanganate. On dissolving the precipitated manganese oxide with sodium bisulphite and dilute sulphuric acid, the product was repeatedly extracted with ether (total 3000 c.c.). The ether solution was concentrated and treated with 20% aqueous solution of sodium carbonate to dissolve out acidic compounds. On evaporating off the ether, 4.6 gr. of the "neutral" substance were obtained. It formed a crystalline mass, and had the saponification

(3) E. F. Armstrong and T. P. Hilditch, *J. Soc. Chem. Ind.*, **44** (1925), 45T.

value 244, the acid value 134, and the iodine value 15. So it contained, besides neutral substance, still an appreciable amount of acidic product. The latter on isolation was found to consist chiefly of white crystals, which melted at 35°C. and had the neutralisation value 279.6. It was recognised to be impure lauric acid.

The soda extract was saponified with caustic potash and on acidifying shaken with ether. The residue obtained on evaporating off the ether was then treated with petroleum ether, and separated into the soluble and insoluble parts in it.

(1) Petroleum Ether Soluble Part. Yield 10.2 gr.; white crystals with a marked odour of lower fatty acids; neutralisation value 304.4. 9 Gr. of this substance were fractionated under 15 mm. pressure as follows:

	Yield (gr.)	Appearance	Neutr. v.
(a) Until 170°C	0.2	Colourless liquid	—
(b) 170–180°C.	6.3	White crystals	308.1
(c) 180–185°C.	1.5	,, ,,	309.6
(d) Residue	0.5	Pale orange-yellow crystals	—

The boiling point of the chief fraction (b) corresponded to those of lauric acid. The high neutralisation value was due to admixed lower acids formed by the secondary reactions. On recrystallising the mixture of (b) and (c) from 70% alcohol by ice-cooling, 4 gr. of the refined substance were obtained. It was white crystals of m.p. 40°C. and had the neutralisation value 283.6. No depression of melting point was observed by the mixed test with lauric acid (Kahlbaum). So the substance was confirmed to be lauric acid $C_{12}H_{24}O_2$ (m.p. 43.6°C., neutr. v. 280.3).

The acids contained in the mother liquor were converted into barium salts and deposited from hot alcohol. The analysis of the salts gave Ba 26.89% (calc. for $(C_{12}H_{23}O_2)_2Ba$, Ba 25.64%) and showed that they consisted mainly of barium laurate.

(2) Petroleum Ether Insoluble Part. Yield 4.9 gr.; white crystals; neutr. value 755.9. On recrystallisation from acetic ether, first crop, 2.3 gr., m.p. 150.5–151°C., neutr. value 772.8; second crop, 0.6 gr., m.p. 148–148.5°C., neutr. value 773.1. So they were recognised to be adipic acid $C_6H_{10}O_4$ (m.p. 151°C., neutr. value 768.2).

The above experiments have shown that the oxidation products of the octadecenoic acid from nigaki oil are lauric and adipic acids, so that its constitution corresponds to those of petroselinic acid as follows:

$$CH_3.(CH_2)_{10}.CH:CH.(CH_2)_4.COOH.$$

Petroselinic acid was discovered by E. Vongerichten and A. Köhler[4] from the seed oil of parsley, *Petroselinum sativum*. The content of the acid in the mixed fatty acids of the oil has been stated to be about 76%. Further the acid occurs in the seed oil of *Hedera helix* (up to 62%).[5]

We have found petroselinic acid in the oil of a tree quite different from the above two plants.

(4) Nigaki Shell and Kernel Oils. Besides the nigaki oil mention-above, we have examined the shell and kernel oils separately.

The shell oil was a brownish-yellow liquid mixed with solid constituents at 20°C.; it became clear on warming to 29°C. It had a bitter taste.

The kernel oil was a yellow liquid at 20°C.; at the room temperature of 13°C., it mostly solidified to a white crystalline mass. The oil had also a bitter taste.

	Shell oil	Kernel oil
Sp. gr.	d_4^{30} 0.9580	d_4^{15} 0.9217
Ref. index	d_D^{30} 1.4710	n_D^{20} 1.4705
Acid value	44.5	4.8
Saponif. value	219.7	190.4
Iodine value (Wijs)	50.6	93.9

The shell oil had a high saponification value and a low iodine value; it appeared to contain chiefly the comparatively lower saturated acids (mainly palmitic). Whether the oil contains petroselinic acid or not, has not yet been ascertained.

The kernel oil contained petroselinic acid as the chief constituent. The ether solution of the lead salts of the fatty acids of the oil gave a large amount of precipitate at 10°C., which did not wholly dissolve at 15°C. The precipitate consisted mostly of lead petroselinate. The liquid acids isolated from the ether-soluble lead salts at 15°C. had the iodine value 104, a fact indicating the occurrence of the higher unsaturated acids (linolic and linolenic) in the oil.

Summary.

(1) The properties and composition of the nigaki oil (a mixture of the shell and kernel oils) obtained from the whole fruit of "nigaki," *Picrasma quassioides Benn.*, have been examined.

(4) *Ber.* **42** (1909), 1638.

(5) Grün-Halden, „Analyse der Fette u. Wachse", II, 324.

(2) As the chief constituent of the fatty acids of the oil, the occurrence of petroselinic acid, $C_{18}H_{34}O_2$ ($\Delta^{6:7}$ octadecenoic acid), has been ascertained.

(3) As the other constituent, palmitic acid has been identified from the saturated acids; lauric acid appears also to be present. Of the unsaturated acids, oleic, linolic, and linolenic acids are likely to be present.

(4) The shell and kernel oils have also been examined separately. The former contains an appreciable proportion of saturated acids, while the latter has been found to contain petroselinic acid as the chief constituent.

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